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Immunopathology and Infectious Disease

Susceptibility of Human Testis to Human Immunodeficiency Virus-1 Infection *in Situ* and *in Vitro*

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Semen represents the main vector for human immunodeficiency virus (HIV) dissemination worldwide and has been shown to harbor replication-competent virus despite otherwise effective highly active anti-retroviral therapy, which achieves undetectable viral load in plasma. Despite this, the origin of seminal HIV particles remains unclear, as does the question of whether the male genital tract organs contribute virus to semen. Here we investigated the presence of HIV receptors within the human testis using immunohistochemistry and quantitative real-time polymerase chain reaction. We also analyzed the infectivity of a dual tropic HIV-1 strain in an organotypic culture, as well as the impact of viral exposure on testosterone production. Our study establishes that CXCR4$^+$, CCR5$^+$, CD4$^+$, and DC-SIGN$^+$ cells are present within the interstitial tissue of human testis and that these molecules persist throughout our organotypic culture. Our data also reveal that the human testis is permissive to HIV-1 and supports productive infection, leaving testosterone production apparently unaffected. Infected cells appeared to be testicular macrophages located within the interstitial tissue. That the testis itself represents a potential source of virus in semen could play a role in preventing viral eradication from semen because this organ constitutes a pharmacological sanctuary for many current antiretrovirals. *(Am J Pathol 2006, 169:2094–2103; DOI: 10.2353/ajpath.2006.060191)*

With sexual contact being the main cause of the spread of human immunodeficiency virus (HIV) and male to female transmission rates being higher and more efficient than female to male, semen represents the foremost vector of HIV dissemination worldwide. However, the origin of the virus in the semen is still unclear. Several arguments point to the existence of local sources producing free viral particles in this bodily fluid. First, a number of studies clearly indicate that semen represents a viral compartment distinct from the blood.[1] Second, seminal leukocytes harbor HIV-1 populations different from those found in the seminal plasma.[2,3] However, it remains to be proven whether and which male genital tract organ(s) contribute virus to semen. Furthermore, the detection of HIV-1 RNA in the semen of highly active anti-retroviral therapy-treated seropositive men, even in the absence of detectable levels of viral RNA in plasma, indicates that the male reproductive tract may constitute a sanctuary for HIV-1 replication.[4–9] Indeed, subtherapeutic concentrations of protease inhibitors are evidenced in semen,[10] and sexual transmission of drug-resistant strains are currently on the rise.[11–13] Few previous studies have sought the presence of HIV in testes from acquired immune deficiency syndrome (AIDS)-deceased men, and what data there are remain controversial,[1] whereas, for obvious ethical reasons, in-depth investigations of the impact of HIV on the testis of asymptomatic men have never been performed. Therefore, in this context, the identification of the sources of HIV and of potential reservoirs in the male genital tract is essential.

In vitro studies using a number of different human organs have proved invaluable for improving the understanding of HIV pathogenesis.[14–23] We have recently

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developed an organotypic culture of human testis[24] with the primary objective of studying HIV infection within a control environment preserving cellular interactions. Using this *in vitro* model, and based on a series of experiments *in situ*, the present study provides a detailed analysis of HIV receptor expression within the testis and investigates the ability of this organ to be infected by a dual-tropic HIV-1 strain *in vitro*.

Materials and Methods

Chemicals and Reagents

The following reagents were used: Dulbecco's modified Eagle's medium, modified Eagle's medium, and RPMI 1640 medium (all from Gibco BRL, Life Technologies, Cergy-Pontoise, France), fetal calf serum (Eurobio, Courtaboeuf, France), glutamine (Gibco BRL), transferrin, insulin, vitamin A, vitamin C, vitamin E, phytohemagglutinin (all from Sigma, Sigma-Aldrich Corp., St. Quentin Fallavier, France), penicillin-streptomycin (Eurobio), recombinant human follicle-stimulating hormone (Puregon, Organon, France), recombinant human chorionic gonadotropin (hCG) (Chorulon; Intervet, Angers, France), and interleukin-2 (Boehringer-Mannheim, Mannheim, Germany).

Antibodies, Plasmids, and Cell Lines

The following antibodies and matching isotype controls were used: mouse monoclonal anti-HLA-DR, anti-CD68, anti-MAC 387 (all from DAKO, Trappes, France), and anti-human CD4 (Novocastra, Newcastle, UK) with mouse IgG1 isotype control (DAKO), mouse anti-human CCR5 (clone 45523) and IgG2b (both R&D Systems, Minneapolis, MN), mouse anti-human CXCR4 (clone 12G5; Dr. J. Hoxie, University of Pennsylvania, Philadelphia, PA, and Centralized Facility for AIDS Reagent supported by EU program EVA/MRC and the UK Medical Research Council) and IgG2a (R&D Systems), rabbit anti-S100, and control serum (DAKO). The previously characterized rabbit anti-human DC-SIGN CSRD[25] was generously provided by Dr. Y. van Kooyk (Free University Medical Center, Amsterdam, The Netherlands), and control rabbit serum was purchased from DAKO. Plasmid encoding human DC-SIGN[26] was kindly provided by Dr. A.L. Corbi (Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Cientificas, Madrid, Spain) and plasmids encoding human CD4, CXCR4, and CCR5 were gifts from Dr. P. Clapham (University of Massachusetts Medical School, Worcester, MA). The pNL4.3 plasmid[27] was provided by F. Mamano (INSERM U552, Paris, France). The 8E5 lymphocytic cell line,[28] which contains an integrated single-copy of reverse transcriptase (RT)-defective HIV, was maintained at 37°C in RPMI 1640 media supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, and antibiotics. The previously described P4P cell line,[29] consisting of $CD4^+CXCR4^+CCR5^+$ HeLa cells carrying the *lacZ* gene under the control of the HIV-1 long terminal repeat, was provided by Dr. P. Charneau (Pasteur Institute, Paris, France) and maintained at 37°C in modified Eagle's medium supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, and antibiotics.

HIV Strain

The HIV-1 strain used in this study was the dual-tropic strain (R5X4) HIV-1 molecular clone 89.6[30] and was obtained from the NIBSC Centralized Facility for AIDS Reagent. HIV-$1_{89.6}$ was grown in peripheral blood mononuclear cells (PBMCs) stimulated by phytohemagglutinin (3 μg/ml) and interleukin-2 (5%), and aliquots of titrated virus stock were stored at −80°C until needed. The infectious titer of virus stock was determined by limiting dilution assays on the above-described P4P cells, and β-galactosidase activity was quantified by the β-Gal gene reporter assay (Roche Applied Sciences, Mannheim, Germany). The infectious titer was expressed as 50% tissue culture infective dose ($TCID_{50}$) per ml calculated using the Reed-Muench formula.[31]

Organotypic Culture of Human Testis Explants

The protocol was approved by the local ethics committee of the University of Rennes, and informed consent was obtained from the donors. Normal testes, obtained from prostate cancer patients, not subjected to any hormone therapy, were transported on ice immediately after orchidectomy in fresh medium. The clinical status of the patients revealed no reproductive abnormalities or testicular infections. The materials were assessed by transluminination for the occurrence of spermatogenesis before their subsequent use, and culture was performed as previously described.[24] In brief, testicular tissue was dissected into 3-mm^3 fragments and two explants transferred onto a polyethylene terephthalate insert in a well of a six-well plate (Falcon Labware; Becton-Dickinson and Co., Lincoln Park, NJ) containing 2 ml of media (Dulbecco's modified Eagle's medium supplemented with antibiotics, 10% fetal calf serum, 1 mmol/L sodium pyruvate, 4 mmol/L glutamine, 100 ng/ml vitamin A, 200 ng/ml vitamin E, 50 ng/ml vitamin C, 10 μg/ml insulin, and 5 μg/ml transferrin). The culture was established for 14 to 16 days in a humidified atmosphere containing 5% CO_2 at 37°C, medium changed every 2 days, and stored frozen at −20°C. As previously reported, adult human testis explants in culture for up to 2 weeks retained overall structural integrity of both the interstitial and the seminiferous tubules compartments, and all germ cell types associated with Sertoli cells presented classic ultrastructural characteristics, testosterone secretion, and expression of somatic cell markers.[24] Every 2 days, testis explants were either fixed in neutral buffered 4% formaldehyde for immunohistochemical analysis or frozen and stored at −80°C until nucleic acid extraction.

Light Microscopy

For histological analysis, testicular explants were fixed in neutral buffered 4% paraformaldehyde for 2 hours at 4°C,

dehydrated by immersion in a series of graduated alcohol concentrations, embedded in paraffin, sectioned at 5.0 μm, and stained with Masson's hemalum for examination using an Olympus AX60TF microscope with monochromatic objectives (Olympus, Paris, France).

Immunostaining and Quantification of HIV Receptor-Bearing Cells

To assess the topographical distribution of HIV target cells in cultured testis explants, immunohistochemistry using avidin-biotin peroxidase complex technique was performed on formaldehyde-fixed, paraffin-embedded tissues. Tissue sections (5 μm) were deparaffinized, rehydrated, and incubated in an antigen-retrieval solution (10 mmol/L citrate, pH 6) for 20 minutes as previously described[32] and then washed in 0.05 mol/L phosphate-buffered saline (PBS, pH 7.6). Endogenous peroxidases were inactivated (PBS-3% H_2O_2 for 5 minutes) and slides saturated with PBS-1% bovine serum albumin, before overnight incubation at 4°C with the antibody or appropriate isotype control or serum diluted in PBS-2% bovine serum albumin [anti-CD4 (16.5 μg/ml), anti-CCR5 (2 μg/ml), anti-CXCR4 (5 μg/ml), anti-DC-SIGN (1/500), anti-S-100 (11.25 μg/ml), anti-HLA DR (0.6 μg/ml), anti-CD68 (1.2 μg/ml), or anti-MAC387 (8.6 μg/ml)]. Subsequent steps were performed at room temperature. Specific binding of the primary antibody was revealed using biotinylated secondary antibodies, peroxidase-conjugated streptavidin, and either amino-ethyl-carbazole or 3,3′ diaminobenzidine substrates. The nuclei were counterstained with Masson's hemalum. The sections were photographed by an Olympus AX60TF microscope with monochromatic objectives (Olympus) coupled to a digital macrocamera (Kigamo, Metis, France). No staining was ever observed with isotype control antibodies or control serum. Stained positive cells were counted in a total of at least 1000 interstitial cells in three independent cultures using the Cast software (Olympus), and their number was expressed as a percentage of total interstitial cells. Colocalization was performed on adjacent histological sections.

Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction (Q-PCR)

Total RNA was extracted from testis explants at days 0, 4, 8, 12, and 16 of culture using the RNeasy isolation kit (Qiagen, Courtaboeuf, France) and depleted of contaminating DNA by DNase treatment (Promega, Charbonnières, France). cDNA was generated from 4 μg of total RNA by using the first strand step of the SuperScript II kit (Invitrogen, Cergy-Pontoise, France). Q-PCR was used to independently determine gene expression levels for CD4, CXCR4, CCR5, and DC-SIGN. PCR was performed on 4 ng and 40 ng of equivalent RNA run in duplicates with the ABI7500 (Applied Biosystems, Foster City, CA) using commercially available master mix and following target probes (Applied Biosystems): Hs00181217_m1 (CD4), Hs00607978_s1 (CXCR4), Hs00152917_m1 (CCR5), Hs00253550_m1 (DC-SIGN), and Hs99999901_s1 (18s). The CT values of each gene was calculated with the ABI Sequence Detection System 1.9 program (Applied Biosystems) and normalized to the level of ribosomal 18s RNA. The gene expression level in three independent cultures at various time points (days 0, 4, 8, 12, and 16) was calculated by the standard curve method using *in vitro*-transcribed RNA from plasmids as absolute standards. Results were expressed as copy numbers of the mRNA of interest per copy of 18s RNA.

HIV Infection of Testicular Explants

Immediately after dissection, two fragments of human testis tissue (~3 mm^3 each) were immersed in 100 μl of cell-free viral supernatant at 10^6 $TCID_{50}$ for 3 hours at 37°C (corresponding to a multiplicity of infection of ~0.1). The explants were then thoroughly rinsed three times in PBS and transferred onto PET inserts (two fragments/insert/well) (Falcon) in six-well plates containing 2 ml of culture medium/well and incubated overnight at 37°C in a humidified atmosphere with 5% CO_2. Culture medium was replaced and collected every 2 days and stored at −80°C for viral RNA extraction, RT assay, and testosterone radioimmunoassay (RIA).

For proviral real-time PCR, explants were treated identically, except that the viral stock was treated with 20 U/ml DNase I at 37°C for 1 hour to eliminate contamination by viral DNA. As a negative control, half of this viral stock was heat-inactivated for 30 minutes at 80°C and used to infect control explants. On days 3, 6, and 12 after infection, individual testis explants were snap-frozen and kept at −80°C until nucleic acid extraction.

RT Activity Assay

For each donor, frozen culture supernatants from two independent wells containing paired explants were assayed in duplicates for RT activity using the Lenti-RT activity assay (Cavidi Tech, Uppsala, Sweden) according to the manufacturer's instructions. Unknown values were obtained from the standard curve interpolation and were expressed as pg/ml of RT.

Viral RNA Quantification by Real-Time Quantitative RT-PCR

Viral RNA was extracted from culture supernatants using the QIAamp viral RNA mini kit (Qiagen) according to the manufacturer's instruction. Quantitative real-time RT-PCR was used to amplify a region with low degree of variability in the long terminal repeat (LTR) gene of the HIV-1 genome as previously described.[33] RT-PCR was performed as a one-step assay on 50 ng of RNA run in duplicate, using commercially available TaqMan one-step RT-PCR MasterMix (Applied Biosystems), with an internal standard for quantification. Amplification and data acquisition were performed with the ABI Prism 7000 sequence de-

tection system (Applied Biosystems). Copy number was calculated based on CT values of the internal standard (the stated linear dynamic range is 55 to 170,000 copies/ml). Two independent wells of the same culture (containing two testis explants each) were analyzed in duplicates. Results from three independent cultures corresponding to different donors are expressed as copy number of HIV RNA per ml of culture supernatant.

Measurement of Proviral HIV-1 DNA Using TaqMan Real-Time PCR

Total DNA was extracted using the QIAamp DNA mini kit (Qiagen) according to the manufacturer's instruction. Quantitative real-time PCR was performed using the above-described primers and probe for HIV-1 and the following primers and probes for albumin gene amplification: Alb-S (5′-GCTGTCATCTCTTGTGGGCTGT-3′), Alb-AS (5′-AAACTCATGGGAGCTGCTGGTT-3′), and Alb TaqMan probe (5′-CCTGTCATGCCCACACAAATCT-CTCC-3′).[34] PCR was performed on 250 ng of DNA and performed as described above. The number of proviral DNA copies in each sample was quantified using calibrated DNA from 8E5 cells. Albumin quantification was used to determine the input level of cellular DNA in the sample, and as an endogenous reference. DNA extracted from explants infected with DNase-treated, heat-inactivated virus was used as a negative control. For each donor and each time point, two separate blocks of tissue were analyzed in duplicate. Results are expressed as HIV DNA copy number/albumin copy number.

Viral Infectivity Assay

Five hundred μl of testis culture supernatant collected at day 14 after infection, or 500 μl of viral stock (10^6 $TCID_{50}$/ml) maintained at 37°C for 14 days (used here as a negative control), were ultracentrifuged for 1 hour at 17,000 rpm. Supernatants were discarded and pellets dissolved in 500 μl of RPMI, which was added to 4×10^6 phytohemagglutinin-activated PBMCs for 2 hours at 37°C. After a 10-minute centrifugation at 1200 rpm, PBMCs were resuspended in 2 ml of RPMI 1640 supplemented with 5% interleukin-2 and maintained at 37°C for 9 days. Culture medium was changed every 3 days and stored frozen at −80°C for subsequent viral RNA extraction and RT assay.

Simultaneous in Situ *Hybridization and Immunohistochemical Staining*

Identification of cell types expressing HIV RNA was performed on the explants at the end of the 2-week culture period by combining *in situ* hybridization for HIV and immunohistochemical staining for cell markers, as previously described.[35] The gag probe comprised a 505-bp PCR fragment from NL4.3 (nucleotides 1483 to 1988, GenBank accession number M19921) subcloned into pCRII-TOPO. ^{35}S-UTP-labeled riboprobes were synthesized by *in vitro* transcription of the linearized plasmid template using T7 or SP6 RNA polymerase to generate the anti-sense or corresponding sense probe. The orientation of the inserted fragment was checked by sequencing. Radioactive *in situ* hybridization was performed as previously described.[36] Tissues were prepared for hybridization by rehydration and treatment with proteinase K (10 mg/ml). To inhibit the endogenous peroxidase activity, tissues were incubated for 10 minutes with 3% H_2O_2 (v/v). Then, unspecific binding sites were blocked with 2% bovine serum albumin in phosphate-buffered saline (PBS), pH 7.4. Incubation with primary anti-CD68 antibodies (final concentration, 1.2 μg/ml) was performed overnight at 4°C. For negative control, tissues were incubated with mouse immunoglobulin (IgG1) (DAKO, Glostrup, Denmark). Subsequently, the following procedures were performed at room temperature. A secondary biotinylated goat anti-mouse IgG1 was applied for 2 hours before incubation with horseradish peroxidase-streptavidin complex for 30 minutes. Between all described steps, slides were thoroughly washed with PBS. Bound antibodies were visualized after diaminobenzidine substrate (DAKO). After acetylation, dehydration, and air-drying, tissue sections were hybridized under coverslips with the radioactive probe diluted in hybridization buffer (4.10^6 cpm riboprobe/slide; 70 μl on each slide) and incubated for 12 to 14 hours at 55°C in a humid chamber. After hybridization, coverslips were removed by bathing in 5× standard saline citrate (SSC: 15 mmol/L sodium citrate, 150 mmol/L NaCl, pH 7) with 10 mmol/L dithiothreitol at 55°C for 30 minutes. The slides were washed twice in 2× SSC, 50% formamide, and 10 mmol/L dithiothreitol at 65°C. After a rinse in NTE buffer (10 mmol/L Tris-HCl, 0.5 mol/L NaCl, and 85 mmol/L ethylenediaminetetraacetic acid, pH 8), sections were treated with RNase A (20 mg/ml NTE, 30 minutes at 37°C) and then rinsed again with NTE buffer before another wash in 2× SSC, 50% formamide, and 10 mmol/L dithiothreitol at 65°C for 30 minutes. Before autoradiography, the tissues were rinsed in 2× SSC (15 minutes) and 0.1× SSC (15 minutes) at room temperature and dehydrated in an ethanol series containing 0.3 mol/L ammonium acetate. Autoradiography was performed by dipping the slides into Ilford K5 nuclear track emulsion. After exposure in the dark for 10 days at 4°C, slides were developed, counterstained with Masson's hemalum solution, and coverslipped in Eukitt (CML, Nemours, France). The slides were observed and photographed with a Provis photomicroscope (Olympus France, Rungis, France). The specificity of the hybridization signal was systematically checked by hybridizing sense probes on parallel sections. $CD68^+$ cells and HIV RNA^+ cells were counted on the same whole slide sections in three independent cultures using the Cast software (Olympus).

Measurement of Testosterone Production

Testosterone secreted into the medium was assayed in duplicates using a commercial RIA based on competitive binding with ^{125}I-labeled testosterone, according to the manufacturer's recommendations (Beckman Coulter,

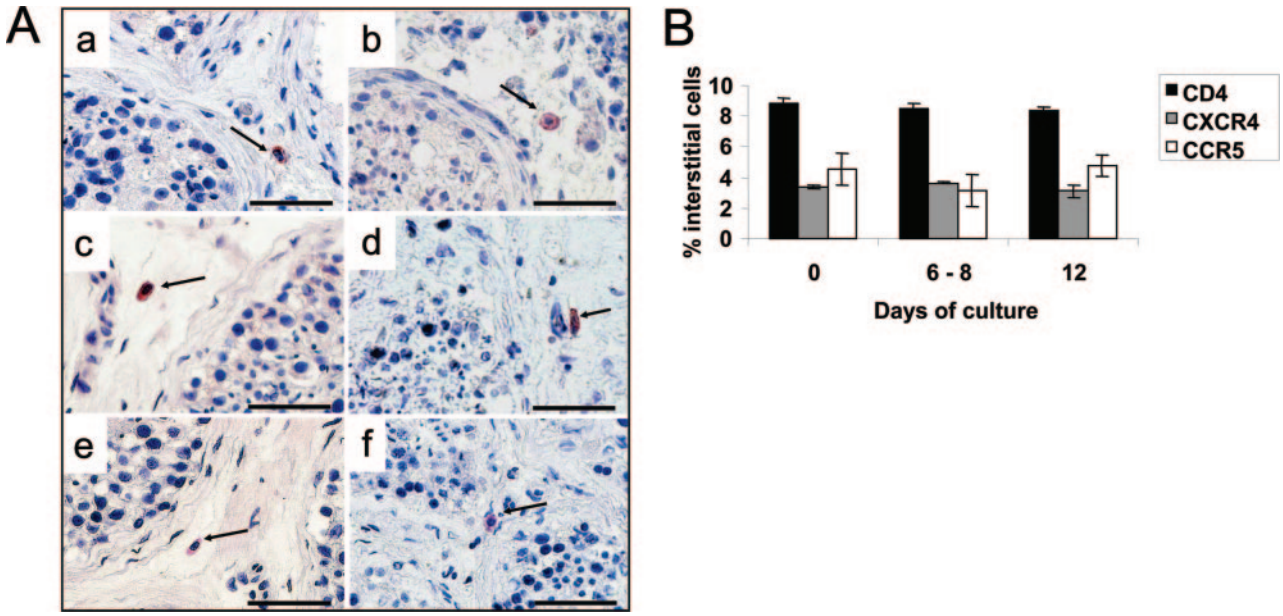

Figure 1. A: Immunohistochemical localization of HIV receptor-bearing cells in human testis and their maintenance *in vitro* throughout the organotypic culture. Paraformaldehyde-fixed paraffin sections were stained with anti-CD4 (**a**, **b**), anti-CXCR4 (**c**, **d**), and anti-CCR5 (**e**, **f**). **a**, **c**, and **e:** Testis sections before culture; **b**, **d**, and **f:** testis sections after 12 days of culture. Representative positive cells are indicated by **arrows**. **B:** Number of CD4-, CXCR4-, and CCR5-labeled cells during the culture of testicular explants. The cells were counted in at least 1000 interstitial cells using the Cast grid software. The average percentages shown correspond to the mean ± SEM of three separate independent experiments corresponding to three donors. Scale bars = 50 μm.

Villepinte, France). Unknown values were obtained from the standard curve interpolation and were expressed in ng/ml.

Statistics

All values (excepting box plot) are the mean ± SEM. The significance of the differences between values was evaluated using Dunnett's test. This test controls the type I experiment-wise error for comparisons of all samples against a control (described in the figure legends). A value of $P < 0.05$ was considered statistically significant.

Results

HIV Receptor Expression within the Testis in Vivo *and in Culture*

Testis explants from three individuals were fixed at day 0, 4, 8, and 12 of culture and stained for potential HIV target cells using antibodies directed against cellular HIV receptors. At day 0 of culture, specific staining for CD4, CXCR4, and CCR5 was consistently observed within the interstitial tissue, although none of these markers were ever detected, neither within the seminiferous tubules nor in the peritubular cells (Figure 1A; a, c, and e). A similar distribution was observed after 12 days of culture (Figure 1A; b, d, and f). At the beginning of the culture, quantitative immunohistochemistry revealed that $CD4^+$ cells represented an average of ~8% of total interstitial cells, whereas $CXCR4^+$ cells represented ~3% of interstitial cells, and $CCR5^+$ cells, ~4% (Figure 1B). That approximately twice as many cells were labeled for CD4 than for the two chemokine receptors probably results from differences in the sensitivity of the antibodies used because similar results were obtained in lymph nodes (data not shown). That the levels of $CD4^+$, $CXCR4^+$, $CCR5^+$, and $DC\text{-}SIGN^+$ were maintained throughout the culture period, indicates the persistence of the main HIV target cell pool in our culture system (Figure 1B). Furthermore, serial sections of testicular tissue were used to locate and then determine the nature of the cell population(s) expressing DC-SIGN. DC-SIGN labeling co-localized with the antigen-presenting cell marker HLA-DR,[37] the myeloid cell marker CD68,[38] and the infiltrated macrophage marker MAC387[39] (Figure 2). In contrast, labeling with the dendritic cell marker S100[40] did not co-localize with DC-SIGN, although $S100^+$ $CD68^+$ cells were observed in the interstitium (Figure 2). The persistence of the main HIV target cell pool in our culture system was further established using real-time quantitative RT-PCR, as the CD4, CXCR4, and CCR5 mRNAs copy numbers were maintained for up to 16 days of culture (Figure 3). The expression level of each of the three mRNAs was ~2 log lower in testicular tissue than in lymph nodes. They displayed the same pattern of expression in both tissues, with CXCR4 being consistently the most abundant and CCR5 generally the least (Figure 3). We also tested for the expression of the DC-SIGN mRNA and evidenced its presence and persistence throughout the culture, with a level of expression close to 1 log lower than in lymph nodes (Figure 3).

Susceptibility of Human Testis to Infection by HIV-1

Reverse-transcriptase activity of testicular tissue exposed to the R5X4 HIV-1 strain 89.6 significantly raised between day 12 and day 14 in the three independent

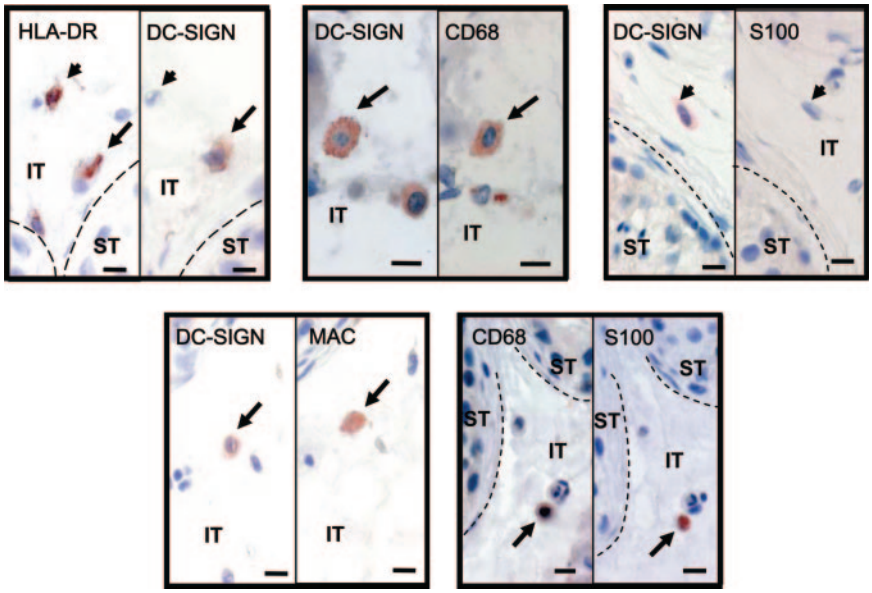

Figure 2. Immunolocalization and co-localization of DC-SIGN, HLA-DR, CD68, S-100, and MAC 387 in the human testis. Paraformaldehyde-fixed paraffin serial sections (each square represents two serial sections) were stained with anti-DC-SIGN, anti-HLA-DR, anti-CD68, anti-S-100, or anti-MAC 387 antibodies, as indicated on the figure. Co-labeled cells are indicated by **solid arrows**, and cells that did not co-label are indicated by **arrowheads**. ST, seminiferous tubules; IT, interstitial tissue. The results shown are representative of three separate independent experiments. Scale bars = 10 μm.

cultures performed, each culture corresponding to a different donor (Figure 4A). Consistent with these results, Q-PCR in the culture supernatants revealed a significant increase in HIV RNA copy number by day 14 after exposure to HIV-1 (Figure 4B). To determine whether the free virus thus far detected was attributable to a low level of viral production by the testis or to the release of viral particles after sequestration or cell death, the quantity of proviral DNA within the testis explants was measured using Q-PCR. HIV-1 proviral DNA level consistently and significantly rose by ~1.5 log between day 3 and day 12 after infection, indicating productive infection (Figure 5). Of note is that, despite the infection, the overall morphology of the testicular explants appeared consistently unaffected (data not shown).

To further test the ability of infected testicular explants to produce infectious progeny HIV virions, we used the day 14 supernatant of the three infected tissue cultures described above, as a virus inoculum to transfer HIV to activated PBMCs. Productive infection of PBMCs was always evidenced by the increase in RT activity in culture supernatants collected between day 3 and day 9 after infection (Figure 6A). The RT activity was associated with an increase of viral RNA copy number in PBMC supernatant (Figure 6B). In contrast, an inoculum of HIV-$1_{89.6}$ left in culture medium for 14 days at 37°C did not lead to productive infection of PBMCs (Figure 6, A and B).

Infected cells were localized within the explants using combined *in situ* hybridization for HIV RNA and immunophenotyping. Infected cells were detected exclusively in

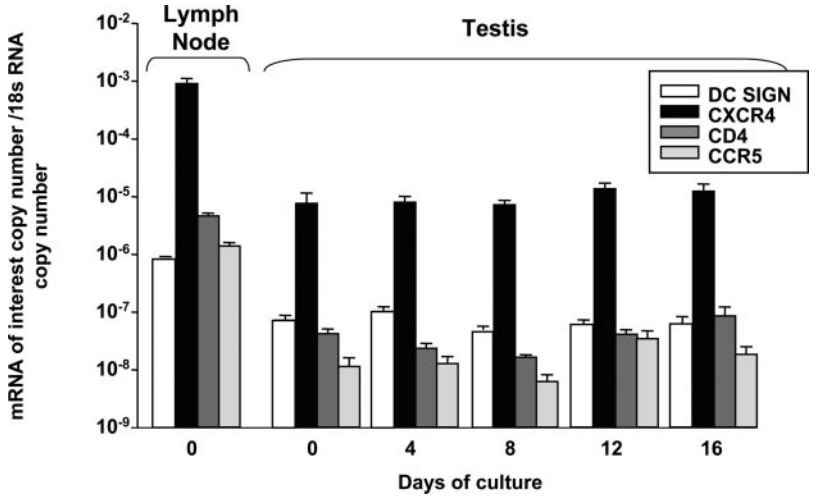

Figure 3. Real-time RT-PCR quantification of transcripts encoding HIV receptors in testis explants before and during culture and comparison with lymph nodes (no culture). The absolute copy number of the cDNA of interest is standardized to the copy number of the ubiquitous 18s housekeeping gene cDNA. The results represent the mean ± SEM of three separate independent experiments corresponding to three donors.

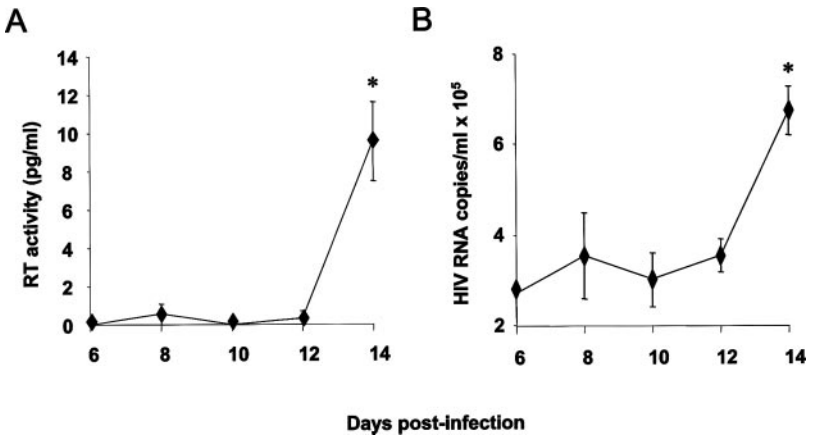

Figure 4. RT activity detected using the Lenti-RT activity assay (**A**) and viral RNA copy number quantified by real-time RT-PCR (**B**) were measured in supernatants of three independent testis organotypic cultures from different donors exposed to HIV-$1_{89.6}$. Each dot represents the mean ± SEM of three independent cultures (Dunnett test, $^*P < 0.05$; control, day 6).

the interstitial tissue (Figure 7, B and C). They systematically co-localized with CD68, a myeloid cell lineage marker (Figure 7C). An average of 1 to 2% of $CD68^+$ cells reacted positively for HIV gag RNA. No significant effect of HIV infection on testosterone production was observed in the two independent cultures tested (data not shown).

Discussion

Although there is little doubt that circulating HIV-infected lymphocytes and macrophages can infiltrate the inflamed testis as part of the overall dissemination of the virus in the later stages of AIDS,[1,41] it remains to be clarified whether the testis can harbor and replicate the virus during the earlier stages of the disease. To date, two studies performed on testicular tissue from HIV^+ asymptomatic men reported the presence of proviral DNA in germ cells[42] and/or in a few lymphomononuclear cells within the interstitium.[2,42] However, no additional experiments of this type have been performed, and some of these findings have been highly criticized.[43] Thus, proof of the susceptibility of testicular cells to HIV during the asymptomatic stage of the infection is still lacking.

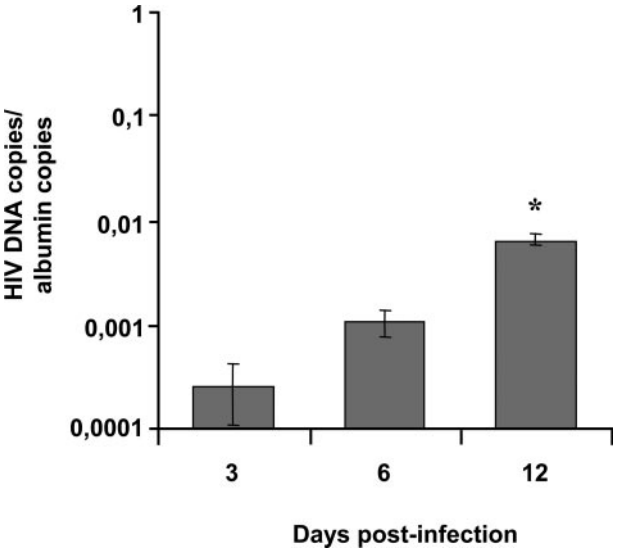

Figure 5. Accumulation of HIV-1 DNA in testis explants at various days after infection with HIV-$1_{89.6}$, as assayed for LTR DNA by quantitative real-time PCR. Data represent the mean ± SEM of two independent experiments using paired explants from separate donors (Dunnett test, $^*P < 0.05$; control, day 3).

The organotypic culture of human testis, which we recently developed and validated,[24] preserves the complex architecture and interactions existing *in vivo* in the male gonad and allows the first *in vitro* analysis of the susceptibility of the testis to HIV infection. Using this culture system, we first confirmed the expression of the HIV receptors CD4, CCR5, and CXCR4 on cells scattered within the testicular interstitium, previously identified as resident macrophages,[44] and showed the maintenance of these receptors throughout the duration of the culture using immunohistochemistry and Q-PCR. In comparison with lymph nodes, the abundance of the three receptor-encoding transcripts appears to be 100-fold lower in the testis.

DC-SIGN binds to HIV with high affinity and facilitates its capture and transmission.[45] We revealed here the presence of the C-type lectin DC-SIGN protein and transcript in the human testis. This contradicts a previous immunohistochemistry study,[46] but this may result from differences in tissue processing or antibody sensitivities. We demonstrate that DC-SIGN co-localized with a subpopulation of HLA DR^+ $CD68^+$ cells expressing MAC387, a marker for resident macrophages. DC-SIGN expression by tissue resident macrophages has previously been described in the lung[46] and the placenta.[47] Recently, DC-SIGN was shown on perivascular macrophages within the central nervous system[48] and macrophages in breast milk,[49] further demonstrating that this receptor is not solely expressed by dendritic cells. Within the testicular interstitial tissue, we have also evidenced a dendritic cell population ($CD68^+S100^+$ cells[50]) that did not express DC-SIGN. This was previously encountered in lymph nodes in which the $S100^+$ cells do not all express DC-SIGN.[25]The presence of DC-SIGN expression by testicular resident macrophages expressing CD4 and co-receptors is likely to enhance their infection in *cis*-but

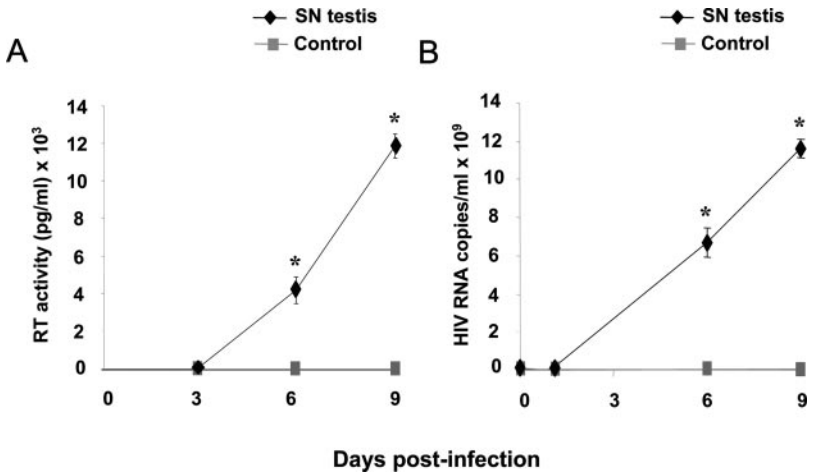

Figure 6. RT activity (**A**) and HIV RNA copy numbers (**B**) measured in supernatants of activated PBMCs exposed to day 14 supernatants of three independent cultures of testicular explants infected with HIV-$1_{89.6}$. Control represents viral stock (10^6 $TCID_{50}$/ml) maintained at 37°C for 14 days before infection of activated PBMC culture (Dunnett test, $^*P < 0.05$; control, day 3 for RT and day 0 for HIV RNA).

also to play a role in enhancing the transmission in *trans* of the bound virus to other susceptible target cells such as, for example, the lymphocytes infiltrating the testis during the disease-induced inflammation.

We next analyzed whether the testis could be productively infected by HIV *in vitro*. Because of the paucity of testicular tissue available and the irregularity of their availability, only one HIV-1 strain was tested in this study. The dual-tropic molecular clone 89.6 used here can infect $CD4^+$ primary and transfected cells *in vitro* through either CCR5 or CXCR4. This clone can also use some others alternate chemokine receptors,[51,52] although its co-receptor usage has been shown to be restricted to CXCR4 in *ex vivo*-infected lymphoid tissue.[53,54] We demonstrate that this viral strain generated a low but reproducible productive infection of the testis from three men. RT activity was first detected in the organotypic culture supernatants between days 12 and 14 after infection and was associated with increased HIV RNA and DNA copies. The produced viral particles subsequently replicated in PBMCs in culture, demonstrating their infectiousness. In line with the low levels and late onset of productive infection observed by PCR, which suggests a restricted number of replicating target cells, *in situ* hybridization for HIV RNA revealed a scarce distribution of productively infected cells in the interstitial tissue. This tissue localization is in agreement with previous findings in asymptomatic individuals.[2] Co-labeling with specific cell markers identified those cells as $CD68^+$ testicular macrophages. In contrast, the relatively high number of proviral DNA copies is suggestive of a latent infection of other cell types, possibly through unidentified receptors.

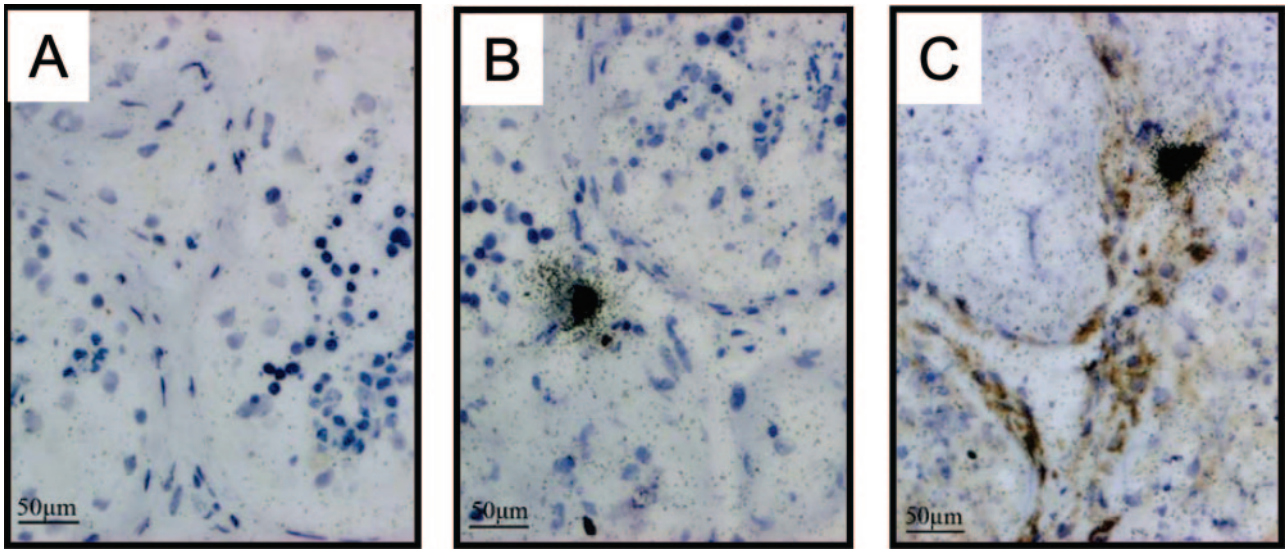

Figure 7. Localization of HIV RNA in testis explants infected for 2 weeks with HIV-$1_{89.6}$, using *in situ* hybridization combined with immunostaining for specific cell markers. **A:** No staining was ever observed in explants hybridized with sense riboprobe and isotypic control antibodies. **B:** Infected cells were detected in the interstitium of explants hybridized with anti-sense riboprobe and isotypic control antibodies. **C:** Combined *in situ* hybridization for viral RNA and immunohistochemistry for CD68 revealed black silver grains clustered over brown cells, indicating productive infection of cells of macrophage lineage. Exposure time was 10 days. Results are representative of three independent experiments performed on separate individuals.

The changes in circulating testosterone concentrations observed in asymptomatic HIV-positive individuals and in AIDS patients[1] prompted us to analyze testosterone production in the testis explants infected *in vitro*. However, under our culture conditions, infection of the testis did not modify testosterone secretion, which is in line with our previous data demonstrating that isolated human Leydig cells are not infected by HIV-1 strains *in vitro*.[55] This contrasts with the situation prevailing in mumps patients as the decrease in circulating testosterone production also observed in these men[56] was associated with a permissivity of human Leydig cells for the mumps virus and a marked reduction of testosterone production *in vitro*.[57]

In conclusion, our results reveal that the testis can sustain productive infection by HIV-1 and that virus-replicating cells within this organ are $CD68^+$ macrophages. Based on our study and those of others,[44] these cells are assumed to bear DC-SIGN and HIV receptors CD4, CXCR4, and CCR5. That the testis can produce infectious viral particles represents a very important finding in the context of highly active anti-retroviral therapy because this organ constitutes a pharmacological sanctuary for many current anti-retrovirals. Thus, it is conceivable that the culture system used here to study the early interaction between HIV and testicular cells could also be used for the assessment of the existing anti-retrovirals and the development of new ones.

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References

1. Dejucq-Rainsford N, Jégou B: Viruses in semen and male genital tissues: consequences for the reproductive system and therapeutic perspectives. Curr Pharm Design 2004, 10:557–575
2. Paranjpe S, Craigo J, Patterson B, Ding M, Barroso P, Harrison L, Montelaro R, Gupta P: Subcompartmentalization of HIV-1 quasispecies between seminal cells and seminal plasma indicates their origin in distinct genital tissues. AIDS Res Hum Retroviruses 2002, 18:1271–1280
3. Ghosn J, Viard JP, Katlama C, de Almeida M, Tubiana R, Letourneur F, Aaron L, Goujard C, Salmon D, Leruez-Ville M, Rouzioux C, Chaix ML: Evidence of genotypic resistance diversity of archived and circulating viral strains in blood and semen of pre-treated HIV-infected men. AIDS 2004, 18:447–457
4. Zhang H, Dornadula G, Beumont M, Livornese L, Van Uitert Jr B, Henning K, Pomerantz RJ: Human immunodeficiency virus type 1 in the semen of men receiving highly active antiretroviral therapy. N Engl J Med 1998, 339:1803–1809
5. Kiessling AA, Fitzgerald LM, Zhang D, Chhay H, Brettler D, Eyre RC, Steinberg J, McGowan K, Byrn RA: Human immunodeficiency virus in semen arises from a genetically distinct virus reservoir. AIDS Res Hum Retroviruses 1998, 14(Suppl 1):S33–S41
6. Mayer KH, Boswell S, Goldstein R, Lo W, Xu C, Tucker L, DePasquale MP, D'Aquila R, Anderson DJ: Persistence of human immunodeficiency virus in semen after adding indinavir to combination antiretroviral therapy. Clin Infect Dis 1999, 28:1252–1259
7. Lafeuillade A, Solas C, Halfon P, Chadapaud S, Hittinger G, Lacarelle B: Differences in the detection of three HIV-1 protease inhibitors in non-blood compartments: clinical correlations. HIV Clin Trials 2002, 3:27–35
8. Solas C, Lafeuillade A, Halfon P, Chadapaud S, Hittinger G, Lacarelle B: Discrepancies between protease inhibitor concentrations and viral load in reservoirs and sanctuary sites in human immunodeficiency virus-infected patients. Antimicrob Agents Chemother 2003, 47:238–243
9. Bujan L, Daudin M, Matsuda T, Righi L, Thauvin L, Berges L, Izopet J, Berrebi A, Massip P, Pasquier C: Factors of intermittent HIV-1 excretion in semen and efficiency of sperm processing in obtaining spermatozoa without HIV-1 genomes. AIDS 2004, 18:757–766
10. Taylor S, Pereira AS: Antiretroviral drug concentrations in semen of HIV-1 infected men. Sex Transm Infect 2001, 77:4–11
11. Little SJ, Holte S, Routy JP, Daar ES, Markowitz M, Collier AC, Koup RA, Mellors JW, Connick E, Conway B, Kilby M, Wang L, Whitcomb JM, Hellmann NS, Richman DD: Antiretroviral-drug resistance among patients recently infected with HIV. N Engl J Med 2002, 347:385–394
12. Grant RM, Hecht FM, Warmerdam M, Liu L, Liegler T, Petropoulos CJ, Hellmann NS, Chesney M, Busch MP, Kahn JO: Time trends in primary HIV-1 drug resistance among recently infected persons. JAMA 2002, 288:181–188
13. Markowitz M, Mohri H, Mehandru S, Shet A, Berry L, Kalyanaraman R, Kim A, Chung C, Jean-Pierre P, Horowitz A, La Mar M, Wrin T, Parkin N, Poles M, Petropoulos C, Mullen M, Boden D, Ho DD: Infection with multidrug resistant, dual-tropic HIV-1 and rapid progression to AIDS: a case report. Lancet 2005, 365:1031–1038
14. Shapshak P, Yoshioka M, Sun NC, Nelson SJ, Rhodes RH, Schiller P, Resnick L, Shah SM, Svenningsson A, Imagawa DT: HIV-1 in postmortem brain tissue from patients with AIDS: a comparison of different detection techniques. AIDS 1992, 6:915–923
15. Fleming SC, Kapembwa MS, MacDonald TT, Griffin GE: Direct in vitro infection of human intestine with HIV-1. AIDS 1992, 6:1099–1104
16. Rosenzweig M, Bunting EM, Damico RL, Clark DP, Gaulton GN: Human neonatal thymic organ culture: an ex vivo model of thymocyte ontogeny and HIV-1 infection. Pathobiology 1994, 62:245–251
17. Margolis LB, Glushakova S, Baibakov B, Zimmerberg J: Syncytium formation in cultured human lymphoid tissue: fusion of implanted HIV glycoprotein 120/41-expressing cells with native CD4+ cells. AIDS Res Hum Retroviruses 1995, 11:697–704
18. Glushakova S, Baibakov B, Zimmerberg J, Margolis LB: Experimental HIV infection of human lymphoid tissue: correlation of CD4+ T cell depletion and virus syncytium-inducing/non-syncytium-inducing phenotype in histocultures inoculated with laboratory strains and patient isolates of HIV type 1. AIDS Res Hum Retroviruses 1997, 13:461–471
19. Reece JC, Handley AJ, Anstee EJ, Morrison WA, Crowe SM, Cameron PU: HIV-1 selection by epidermal dendritic cells during transmission across human skin. J Exp Med 1998, 187:1623–1631
20. Palacio J, Souberbielle BE, Shattock RJ, Robinson G, Manyonda I, Griffin GE: In vitro HIV1 infection of human cervical tissue. Res Virol 1994, 145:155–161
21. Collins KB, Patterson BK, Naus GJ, Landers DV, Gupta P: Development of an in vitro organ culture model to study transmission of HIV-1 in the female genital tract. Nat Med 2000, 6:475–479
22. Fazely F, Fry GN, Thirkill TL, Hakim H, King BF, Douglas GC: Kinetics of HIV infection of human placental syncytiotrophoblast cultures: an ultrastructural and immunocytochemical study. AIDS Res Hum Retroviruses 1995, 11:1023–1030
23. Patterson BK, Landay A, Siegel JN, Flener Z, Pessis D, Chaviano A, Bailey RC: Susceptibility to human immunodeficiency virus-1 infection of human foreskin and cervical tissue grown in explant culture. Am J Pathol 2002, 161:867–873
24. Roulet V, Denis H, Staub C, Le Tortorec A, Delaleu B, Satie AP, Patard JJ, Jégou B, Dejucq-Rainsford N: Human testis in organotypic culture: application for basic or clinical research. Hum Reprod 2006, 21:1564–1575
25. Engering A, van Vliet SJ, Hebeda K, Jackson DG, Prevo R, Singh SK, Geijtenbeek TB, van Krieken H, van Kooyk Y: Dynamic populations of dendritic cell-specific ICAM-3 grabbing nonintegrin-positive immature dendritic cells and liver/lymph node-specific ICAM-3 grabbing nonintegrin-positive endothelial cells in the outer zones of the paracortex of human lymph nodes. Am J Pathol 2004, 164:1587–1595
26. Relloso M, Puig-Kroger A, Pello OM, Rodriguez-Fernandez JL, de la Rosa G, Longo N, Navarro J, Munoz-Fernandez MA, Sanchez-Mateos P, Corbi AL: DC-SIGN (CD209) expression is IL-4 dependent and is

negatively regulated by IFN, TGF-beta, and anti-inflammatory agents. J Immunol 2002, 168:2634–2643
27. Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A, Martin MA: Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J Virol 1986, 59:284–291
28. Folks TM, Justement J, Kinter A, Dinarello CA, Fauci AS: Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. Science 1987, 238:800–802
29. Barin F, Brunet S, Brand D, Moog C, Peyre R, Damond F, Charneau P, Barre-Sinoussi F: Interclade neutralization and enhancement of human immunodeficiency virus type 1 identified by an assay using HeLa cells expressing both CD4 receptor and CXCR4/CCR5 coreceptors. J Infect Dis 2004, 189:322–327
30. Collman R, Balliet JW, Gregory SA, Friedman H, Kolson DL, Nathanson N, Srinivasan A: An infectious molecular clone of an unusual macrophage-tropic and highly cytopathic strain of human immunodeficiency virus type 1. J Virol 1992, 66:7517–7521
31. Krah DL: A simplified multiwell plate assay for the measurement of hepatitis A virus infectivity. Biologicals 1991, 19:223–227
32. Cuevas EC, Bateman AC, Wilkins BS, Johnson PA, Williams JH, Lee AH, Jones DB, Wright DH: Microwave antigen retrieval in immunocytochemistry: a study of 80 antibodies. J Clin Pathol 1994, 47:448–452
33. Rouet F, Ekouevi DK, Inwoley A, Chaix ML, Burgard M, Bequet L, Viho I, Leroy V, Simon F, Dabis F, Rouzioux C: Field evaluation of a rapid human immunodeficiency virus (HIV) serial serologic testing algorithm for diagnosis and differentiation of HIV type 1 (HIV-1), HIV-2, and dual HIV-1-HIV-2 infections in West African pregnant women. J Clin Microbiol 2004, 42:4147–4153
34. Desire N, Dehee A, Schneider V, Jacomet C, Goujon C, Girard PM, Rozenbaum W, Nicolas JC: Quantification of human immunodeficiency virus type 1 proviral load by a TaqMan real-time PCR assay. J Clin Microbiol 2001, 39:1303–1310
35. Fallert BA, Reinhart TA: Improved detection of simian immunodeficiency virus RNA by in situ hybridization in fixed tissue sections: combined effects of temperatures for tissue fixation and probe hybridization. J Virol Methods 2002, 99:23–32
36. Menuet A, Anglade I, Flouriot G, Pakdel F, Kah O: Tissue-specific expression of two structurally different estrogen receptor alpha isoforms along the female reproductive axis of an oviparous species, the rainbow trout. Biol Reprod 2001, 65:1548–1557
37. Thomas A, Lindsay J, Wilkinson M, Bodmer J: HLA-D region alpha-chain monoclonal antibodies: cross-reaction between an anti-DP alpha-chain antibody and smooth muscle. J Pathol 1988, 154:353–363
38. Pulford KA, Sipos A, Cordell JL, Stross WP, Mason DY: Distribution of the CD68 macrophage/myeloid associated antigen. Int Immunol 1990, 2:973–980
39. Flavell DJ, Jones DB, Wright DH: Identification of tissue histiocytes on paraffin sections by a new monoclonal antibody. J Histochem Cytochem 1987, 35:1217–1226
40. Hagen EC, Vennegoor C, Schlingemann RO, van der Velde EA, Ruiter DJ: Correlation of histopathological characteristics with staining patterns in human melanoma assessed by (monoclonal) antibodies reactive on paraffin sections. Histopathology 1986, 10:689–700
41. Dejucq N, Jegou B: Viruses in the mammalian male genital tract and their effects on the reproductive system. Microbiol Mol Biol Rev 2001, 65:208–231
42. Muciaccia B, Filippini A, Ziparo E, Colelli F, Baroni CD, Stefanini M: Testicular germ cells of HIV-seropositive asymptomatic men are infected by the virus. J Reprod Immunol 1998, 41:81–93
43. Pudney J, Nguyen H, Xu C, Anderson DJ: Microscopic evidence against HIV-1 infection of germ cells or attachment to sperm. J Reprod Immunol 1998, 44:57–77
44. Habasque C, Aubry F, Jegou B, Samson M: Study of the HIV-1 receptors CD4, CXCR4, CCR5 and CCR3 in the human and rat testis. Mol Hum Reprod 2002, 8:419–425
45. Turville S, Wilkinson J, Cameron P, Dable J, Cunningham AL: The role of dendritic cell C-type lectin receptors in HIV pathogenesis. J Leukoc Biol 2003, 74:710–718
46. Soilleux EJ, Morris LS, Leslie G, Chehimi J, Luo Q, Levroney E, Trowsdale J, Montaner LJ, Doms RW, Weissman D, Coleman N, Lee B: Constitutive and induced expression of DC-SIGN on dendritic cell and macrophage subpopulations in situ and in vitro. J Leukoc Biol 2002, 71:445–457
47. Soilleux EJ, Morris LS, Lee B, Pohlmann S, Trowsdale J, Doms RW, Coleman N: Placental expression of DC-SIGN may mediate intrauterine vertical transmission of HIV. J Pathol 2001, 195:586–592
48. Fabriek BO, Van Haastert ES, Galea I, Polfliet MM, Dopp ED, Van Den Heuvel MM, Van Den Berg TK, De Groot CJ, Van Der Valk P, Dijkstra CD: CD163-positive perivascular macrophages in the human CNS express molecules for antigen recognition and presentation. Glia 2005, 51:297–305
49. Satomi M, Shimizu M, Shinya E, Watari E, Owaki A, Hidaka C, Ichikawa M, Takeshita T, Takahashi H: Transmission of macrophage-tropic HIV-1 by breast-milk macrophages via DC-SIGN. J Infect Dis 2005, 51:297–305
50. Knight SC: Veiled cells—"dendritic cells" of the peripheral lymph. Immunobiology 1984, 168:349–361
51. Doranz BJ, Rucker J, Yi Y, Smyth RJ, Samson M, Peiper SC, Parmentier M, Collman RG, Doms RW: A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. Cell 1996, 85:1149–1158
52. Yi Y, Rana S, Turner JD, Gaddis N, Collman RG: CXCR-4 is expressed by primary macrophages and supports CCR5-independent infection by dual-tropic but not T-tropic isolates of human immunodeficiency virus type 1. J Virol 1998, 72:772–777
53. Glushakova S, Yi Y, Grivel JC, Singh A, Schols D, De Clercq E, Collman RG, Margolis L: Preferential coreceptor utilization and cytopathicity by dual-tropic HIV-1 in human lymphoid tissue ex vivo. J Clin Invest 1999, 104:R7–R11
54. Malkevitch N, McDermott DH, Yi Y, Grivel JC, Schols D, De Clercq E, Murphy PM, Glushakova S, Collman RG, Margolis L: Coreceptor choice and T cell depletion by R5, X4, and R5X4 HIV-1 variants in CCR5-deficient (CCR5delta32) and normal human lymphoid tissue. Virology 2001, 281:239–247
55. Willey S, Roulet V, Reeves JD, Kergadallan M-L, Thomas E, McKnight A, Jégou B, Dejucq-Rainsford N: Human Leydig cells are productively infected by some HIV-2 and SIV strains but not by HIV-1. AIDS 2003, 17:183–188
56. Aiman J, Brenner PF, MacDonald PC: Androgen and estrogen production in elderly men with gynecomastia and testicular atrophy after mumps orchitis. J Clin Endocrinol Metab 1980, 50:380–386
57. Le Goffic R, Mouchel T, Ruffault A, Patard JJ, Jegou B, Samson M: Mumps virus decreases testosterone production and gamma interferon-induced protein 10 secretion by human Leydig cells. J Virol 2003, 77:3297–3300